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Hpf2 Glycan Structure Is Critical for Protection against Protein Haze Formation in White Wine

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Grape-derived proteins can form haze in wine. Some cell-wall glycoproteins of *Saccharomyces cerevisiae* are capable of reducing protein haze formation. The basis of their haze protective activity is not yet understood. One of the *S. cerevisiae* cell-wall proteins, Hpf2, was produced in *Pichia pastoris*. An altered glycan structure in the *P. pastoris*-produced protein was associated with decreased solubility in water and reduced capacity to mitigate haze formation compared to native Hpf2 protein from *S. cerevisiae*. α-1,2-Linked mannose in the glycan chain was shown to be required for haze protective activity using a series of *S. cerevisiae* deletion mutants (*mnn1*-Δ, *mnn2*-Δ, *mnn4*-Δ, and *mnn5*-Δ), defective in different aspects of glycan processing. The effect of media additives phthalate, casamino acids, and yeast nitrogen base on Hpf2 production in *P. pastoris* were also evaluated. Casamino acids were shown to suppress Hpf2 production in *P. pastoris*.

KEYWORDS: Hpf2; mannoprotein; wine protein haze; glycosylation; *Pichia pastoris*; phthalate; *MNN1*; *MNN2*; *MNN4*; *MNN5*

INTRODUCTION

Protein haze formation in white wine is largely due to the instability of grape-derived pathogenesis-related (PR) proteins (*1*,*2*). Recently, some authors have suggested that sulfate anions play a key factor in stimulating the denaturation of PR proteins, leading to the formation of protein aggregates large enough to be visually detected as haze (*3*). Removal of PR proteins prior to aggregate formation using bentonite is the method currently employed by the wine industry to prevent haze formation (*4*). Because of the costs and quality loss associated with the use of bentonite, finding alternatives for white wine stabilization are areas of research interest (*5*−*10*).

Yeast mannoproteins have a range of oenological functions (reviewed in ref *11*). Three yeast mannoproteins, Hpf1 (*HPF1*, YOL155C), Hpf2 (*PST1*, YDR055W), and invertase (*SUC2*, YIL162W), have been demonstrated to increase perceived grape-derived protein stability in wine (*12*−*14*) by reducing protein aggregate particle size (*15*). However, these mannoproteins have not been observed as components of haze particles. It has been suggested that they act to reduce haze particle size by competing for other factors in wine involved in protein aggregation (*16*). To date, however, their mechanism of action remains unknown.

In this work, the yeast *Pichia pastoris* is investigated as an alternative production host for Hpf2. This organism has many desirable attributes relating to recombinant protein production (*17*,*18*); however, of particular relevance to this work is the contrasting glycosylation profile of proteins secreted by *P. pastoris* compared to those of *Saccharomyces cerevisiae*. Conditions for efficient production of Hpf2 in *P. pastoris* and its purification are explored. Finally, we compare the haze protective activity of *P. pastoris*-produced protein to that of the *S. cerevisiae*-derived form and that produced in a series of *S. cerevisiae* mutants (*mnn1*-Δ, *mnn2*-Δ, *mnn4*-Δ, and *mnn5*-Δ) defective in different aspects of glycan processing (*19*−*21*).

MATERIALS AND METHODS

Materials and Recombinant Host Strains. *P. pastoris* strain X-33, vectors pPICz and pPICzα, and Zeocin from Invitrogen (Carlsbad, CA) were used for recombinant protein expression. Yeast was maintained on 1% (w/v) yeast extract, 2% (w/v) bacteriological peptone, and 2% (w/v) dextrose (YPD). *Escherichia coli* DH10β was used for plasmid preparation, maintenance, and amplification and was maintained on

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Luria broth. Oligonucleotides and Phusion DNA polymerase (Finnzymes) were purchased from GeneWorks (Adelaide, Australia). Other DNA-modifying enzymes, protein-modifying enzymes, and prestained molecular-weight protein standards were purchased from New England Biolabs (Ipswich, MA). Anti-tetra-His antibody was from Qiagen (Doncaster, Australia); horseradish-peroxidase (HRP)-conjugated anti-mouse secondary antibody was from Selinus (Melbourne, Australia); Western Lighting HRP substrate was from Perkin-Elmer (Wellesley, MA); and XK 26/100 column, Hybond ECL nitrocellulose, and Ni^{2+}-Sepharose 6 Fast Flow were from GE Life Sciences (Castle Hill, Australia). GenePulser II, electrophoretic equipment, Sypro Ruby protein stain, and VersaDoc imaging system were from BioRad (Regents Park, Australia). All other reagents were purchased from Ajax Finechem (Taren Point, Australia), Sigma (Castle Hill, Australia), or Difco (Franklin Lakes, NJ).

Expression Vector Construction. pPICzα-HPF2NHis and pPICzα-HPF2CHis were constructed to express *HPF2* in *P. pastoris*. The structures of these constructs are shown diagrammatically in **Figure 1A**. *HPF2NHis* and *HPF2CHis* encoding N- and C-terminally tagged Hpf2 protein, respectively, were amplified by the polymerase chain reaction (PCR) using the primer pairs HPF4F (ctctcgagaaaagagaggctgaagctcatcatcatcatcatcatcatgctacttcctcttcttccagcatac)/HPF2R (ctggcggccgcttacattatagacatgatgattg) and HPF5F (ctctcgagaaaagagaggctgaagctgctacttcctcttcttccagc)/HPF3R (ctggcggccgctcaatgatgatgatgatgatgattgcctttggaactcttagagctagag) from plasmid templates HPF2pYES/GS and p415GAL1HPF2, respectively. The templates have previously been described (*12*). These amplifications resulted in incorporation of *Xho*I and *Not*I restriction sites into 5′ and 3′ ends of the *HPF2* open-reading frame. Amplification products were digested with *Xho*I and *Not*I and ligated into similarly digested vector, pPICzα, using standard methods (*22*). Ligation products were used to transform *E. coli* DH10*β* by electroporation, and plasmid-containing colonies were isolated following plating onto 0.025 gL^{-1} Zeocin. *HPF2* sequence integrity was verified by sequence analysis.

Transformation of *P. pastoris*. Plasmid DNA of pPICzα-HPF2NHis and pPICzα-HPF2fCHis were linearized by digestion with *Pme*I and then introduced into *P. pastoris* strain X-33 by electroporation using a GenePulser II, as described by Cregg and Russell (*23*). Transformants were selected on YPD agar plates containing 0.1 gL^{-1} Zeocin after 1 h of incubation in 1 M sorbitol. Single colonies were screened for construct integration by PCR. Positive integrants were screened for expression in 5 mL shake flask cultures grown in BMMY [10 gL^{-1} yeast extract, 20 gL^{-1} bacto-peptone, 100 mM potassium phosphate at pH 6.0, 13.4 gL^{-1} yeast nitrogen base, 0.04 mgL^{-1} biotin, and 0.5% (v/v) methanol] at 30 °C and 200 rpm for 48 h.

Polyacrylamide Gel Electrophoresis (PAGE), Western Blotting, and Chemiluminescent Detection. Protein was separated on reducing 10% (w/v) acrylamide/Bis sodium dodecyl sulfate (SDS−PAGE) gels by the method of Laemmli (*24*). Silver staining of gels was performed as described in ref *25*. For Western blotting, separated protein was transferred to nitrocellulose essentially as described by Towbin et al. (*26*). Transfer was routinely performed at 80 V for 2 h in a Tris/glycine transfer buffer without methanol. Membranes were blocked with 5% (w/v) skim milk powder in 20 mM Tris at pH 7.4, 0.1% (v/v) Tween 20, and 150 mM NaCl (TBST), incubated with 1:2000 dilution of anti-tetra-His antibody in blocking buffer for 1 h, and washed twice in blocking buffer. They were further incubated with a 1:2000 dilution of anti-mouse HRP-conjugated secondary antibody for 30 min and twice washed in TBST. Membranes were exposed to chemiluminescent substrate for 5 min, and immuno-reactive bands were visualized using a VersaDoc Imaging System (BioRad).

Production of Recombinant Hpf2 and Its Purification. Overnight cultures were prepared by inoculating a single colony of *P. pastoris* transformants into 15 mL of BMGY medium (10 gL^{-1} yeast extract, 20 gL^{-1} bacto-peptone, 100 mM potassium phosphate at pH 6.0, 13.4 gL^{-1} yeast nitrogen base, 0.04 mgL^{-1} biotin, and 10 gL^{-1} glycerol) in a disposable sterile 50 mL centrifuge tube and incubating overnight at 30 °C with shaking. An overnight culture (5 mL) was used to inoculate 250 mL of BMGY medium, which was grown for a further 28 h at 30 °C with shaking. Biomass was harvested by centrifugation for 5 min at 5000*g*. Pelleted cells were resuspended in 500 mL of BMMY medium or a modified basal salt (BS) medium for induction of *HPF2*. BS medium was made according to the modification suggested by Plantz et al. (*27*) and contained 4.3 gL^{-1} KH_2PO_4, 5 gL^{-1} $(NH_4)_2SO_4$, 1.4 gL^{-1} K_2SO_4, 0.8 gL^{-1} $MgSO_4 \cdot 7H_2O$, and 40 gL^{-1} glycerol. Modified trace element solution (PTM4) contained 1.0 gL^{-1} $CuSO_4 \cdot 5H_2O$, 0.08 gL^{-1} NaI, 3.0 gL^{-1} $MnSO_4 \cdot H_2O$, 0.2 gL^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.02 gL^{-1} H_3BO_3, 0.5 gL^{-1} $CaSO_4 \cdot 2H_2O$, 0.5 gL^{-1} $CoCl_2$, 0.14 gL^{-1} $ZnCl_2$, 22 gL^{-1} $FeSO_4 \cdot 7H_2O$, and 0.2 gL^{-1} biotin, and H_2SO_4 was added at 0.12% (v/v) of medium. Unless otherwise specified, the induction period was for 4 days with daily addition of methanol to 0.5% (v/v). Culture media were separated from cellular material by centrifugation at 5000*g*. Cell-free culture supernatants containing recombinant protein were stored at −80 °C until required.

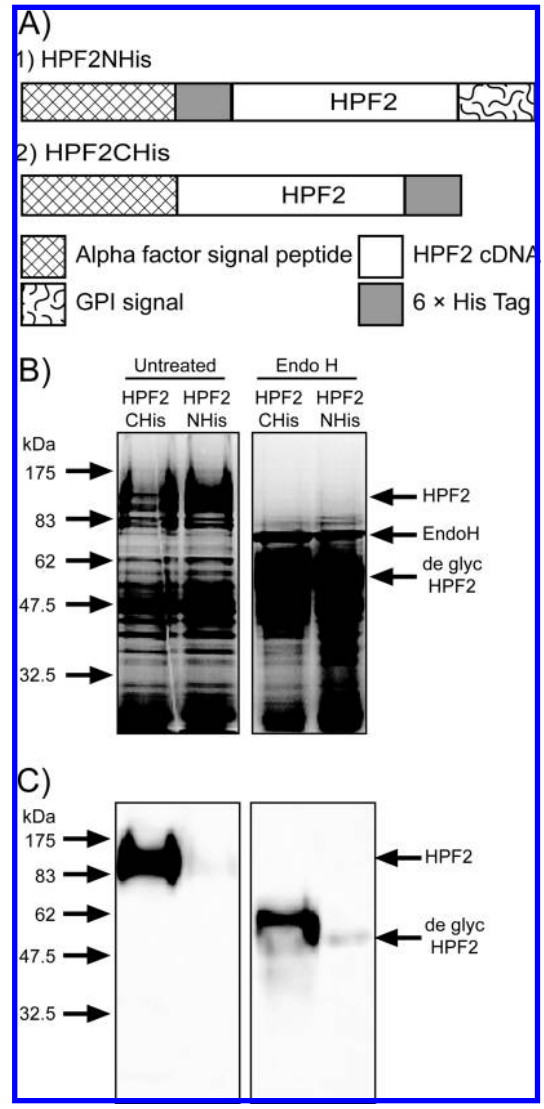

Figure 1. Production and detection of N- and C-terminally tagged versions of Hpf2. (**A**) The 1335 bp *Hpf2* cDNA was expressed as a fusion with α-factor signal peptide. A 6× His tag was engineered into either the N or C terminus (A1 and A2, respectively). In the case of N-terminally tagged Hpf2, the native C terminus encoding a GPI anchor addition site was maintained. The impact on expression and detection of these structural modifications were tested by (**B**) silver-stained SDS−PAGE and (**C**) Western blot with anti-His antibody. The total protein from equal volumes of culture supernatant following induction with methanol for 2 days was TCA-precipitated. Prior to precipitation, samples were either treated with or without endoglycosidase H (Endo H) to remove high mannose structures from protein. Arrows indicate positions of Hpf2 and deglycosylated Hpf2 on the silver-stained gel. Both panels in B and C were from the same gel and membrane, respectively.

Batch binding to Ni^{2+}-sepharose was used to capture His-tagged protein from cell-free supernatants. Cell-free supernatants were mixed in a 5:1 ratio with 5× binding buffer (100 mM Tris at pH 8.0 and 1.5 M NaCl), adjusted to pH 8.0, and mixed with 25 mL of Ni^{2+}-sepharose equilibrated in 1× binding buffer. The suspension was incubated on a rotating platform for 1 h at room temperature (~21 °C) and centrifuged at 1000*g* for 5 min; the supernatant was removed; and the Ni^{2+}-sepharose was resuspended in 1× binding buffer. The suspension was loaded into an XK 26/100 column; the resin was washed with 5 volumes of 10 mM Tris at pH 8.0, 300 mM NaCl, and 20 mM imidazole; and bound protein was eluted with 10 mM Tris at pH 8.0, 300 mM NaCl, and 250 mM imidazole. Eluted material was dialysed against 5 L of water at 4 °C. In total, the water was changed 8 times, with the last incubation taking place overnight. Dialysed material was freeze-dried using a Dynavac FDL 6 freeze-drier unit according to the instructions of the manufacturer. Freeze-dried protein was weighed, dissolved in water, and adjusted to a concentration of 1 gL^{-1} for use in haze protection assays. *S. cerevisiae*-produced Hpf2 was produced and purified as previously described (*12*). The strain backgrounds used for production of Hpf2 with altered glycosylation profiles were obtained from the Euroscarf collection and had the following genotypes: *mnn1*-Δ, BY4741; *MAT a*; *his3D1*; *leu2D0*; *met15D0*; *ura3D0*; YER001w::*kanMX4*; *mnn2*-Δ, BY4742; *MAT a*; *his3D1*; *leu2D0*; *lys2D0*; *ura3D0*; YBR015c::*kanMX4*; *mnn4*-Δ, BY4741; *MAT a*; *his3D1*; *leu2D0*; *met15D0*; *ura3D0*; YKL201c::*kanMX4*; *mnn5*-Δ, BY4741; *MAT a*; *his3D1*; *leu2D0*; *met15D0*; *ura3D0*; YJL186w::*kanMX4*.

Optical density at 600 nm (OD_{600}) was used to estimate biomass accumulation as dry cell weight (DCW). This was achieved by transforming OD_{600} data to DCW using the formula DCW = OD_{600} × 0.64246 (R^2 = 0.9654). The conversion factor was derived from a standard curve of OD_{600} versus DCW generated following the direct measurement of DCW using a moisture balance (AMB50, Inscale Measurement Technology Ltd., Sussex, U.K.).

Endoglycosidase H Treatment of Medium Supernatants from Induced Cultures. Protein from culture supernatants was precipitated by the addition of an equal volume of 20% (v/v) trichloroacetic acid followed by centrifugation at 10000*g* for 10 min. Protein pellets were resuspended in 13.5 μL of autoclaved distilled water. Samples were heated at 98 °C for 10 min following the addition of 1.5 μL of 10× denaturing buffer [5.0% (w/v) SDS and 40 mM DTT]. Samples were allowed to cool, and 1.5 μL of 10× reaction buffer (500 mM sodium citrate at pH 5.5) was added, followed by the addition of 1500 units of Endo H enzyme (New England Biolabs). Protein was deglycosylated by incubation at 37 °C for 4 h. The reaction was halted by the addition of 22 μL of Laemmli sample buffer, followed by heating at 95 °C for 5 min.

Densitometry and Comparison of Hpf2 Yields. After separation of equal volumes of media supernatants by 10% (w/v) SDS−PAGE, gels were soaked overnight in Sypro Ruby, destained for 1 h in 10% (v/v) acetic acid and 10% (v/v) methanol, and photographed on a Versadoc imaging system using settings recommended by the manufacturer. Lane traces were background-corrected, and peaks were identified in Origin (Microcal Software). Areas under intensity curves were determined for Hpf2 bands and divided by areas under intensity curves for 270 ng of purified Hpf2 that was run on each gel. Normalized Hpf2 intensity data were used to compare amounts of Hpf2 produced under different media conditions and between replicates estimated on separate gels.

Haze Protection Assay. Heat haze assays were performed using a modification of the Pocock and Rankine (*28*) heat test described by Waters et al. (*29*). The wine used for studies of *S. cerevisiae* glycosylation mutants was a 2001 Semillon that had been cold-settled at 4 °C for 1 month until yeast collected at the bottom of the tank. Settled wine was racked off and filtered (0.2 μm) into bottles after the addition of potassium metabisulfite to a concentration between 25 and 30 mgL^{-1} free sulfur dioxide. All wine was stored at 4 °C in darkness until required. The wine used for all other studies was a 2004 Sauvignon blanc prepared in the same manner. Neither wine had been bentonite-fined and contained grape pathogenesis-related proteins (125 mgL^{-1} thaumatin-like proteins and 25 mgL^{-1} chitinases). Hpf2 was solubilized in either water, 20 mM citrate at pH 4.0, or wine and centrifuged prior to use. Soluble Hpf2 was quantified by absorbance at 280 nm using experimentally determined extinction coefficients of $\varepsilon^{mg/mL} = 0.2727$ for *S. cerevisiae*-produced Hpf2 and $\varepsilon^{mg/mL} = 0.5858$ for *P. pastoris*-produced Hpf2. Solutions of Hpf2 were added to wine in a 1:10 (v/v) ratio. Haze protective activity of Hpf2 preparations is shown as a percentage of haze formation compared to wine without Hpf2 addition.

Linkage Analysis. Monosaccharide linkage analysis was performed by methylation analysis using sodium hydroxide and methyl iodide in dimethyl sulfoxide as described by Ciucanu and Kerek (*30*). Permethylated alditol acetates were generated by trifluoroacetic acid hydrolysis, reduction with sodium tetradeuteroborate, and acetylation, essentially following the protocol described by Sims and Bacic (*31*). The alditol acetates and the permethylated alditol acetates were recovered in CH_2Cl_2 and separated on a Hewlett-Packard HP 6890 gas chromatograph equipped with an autosampler on a CPSil5 (25 m × 0.3 mm, Chrompack, Middelburg, The Netherlands) column. They were detected by electron impact ionization (70 eV) mass spectrometry on a Hewlett-Packard 5973 mass-selective detector. Data were acquired in full-scan mode [total ion chromatogram (TIC)] to detect ions from *m*/*z* 100−700.

RESULTS

α-Factor Signal Peptide Affects Efficient Secretion of Hpf2 in *P. pastoris*. The ability of different signal peptides to support translation and secretion in *P. pastoris* is protein-dependent (*32*−*34*). Protein secretion using the homologous signal peptide was attempted without success (data not shown). Hpf2 was subsequently produced as a fusion with α-factor signal peptide using constructs *HPF2NHis* and *HPF2CHis*, respectively (**Figure 1A**). The presence of Hpf2 in SDS−PAGE-separated culture supernatants was determined by silver-staining (**Figure 1B**) and Western blotting (**Figure 1C**). Substantial amounts of silver-stained protein can be seen **Figure 1B**, the molecular weight of which is consistent with the size of immuno-reactive Hpf2CHis (**Figure 1C**).

His-Tag Position Impacts on Detection of Hpf2 by Western Blot. The effect of N- and C-terminal 6× His-tag fusion was compared using constructs *HPF2NHis* and *HPF2CHis*, respectively. Hpf2NHis was not readily detectable by Western blot (**Figure 1C**), even though a greater amount of secreted protein is evident in silver-stained gels of supernatants from *HPF2NHis* transformants compared to *HPF2CHis* transformants (**Figure 1B**). The poor detection efficiency of Hpf2NHis protein may be due to either a missing His tag on a proportion of the protein or steric hindrance from the protein structure, preventing access of antibody to the tag. Low detection efficiency of tagged protein in Western blots have been reported for *S. cerevisiae*-produced Hpf1 but not Hpf2 (*12*). It is feasible that antibody access could be impeded by glycosyl addition at the molecules N terminus, and this possibility was subsequently tested. Because of its superior immuno-reactivity, Hpf2CHis was used in the remainder of the work and will be referred to as Hpf2-Pp.

Medium Composition Effects Hpf2 Yield in *P. pastoris*. It has previously been shown that medium composition can have a large impact on recombinant protein accumulation (*35*−*37*). The impact of medium composition on Hpf2-Pp yield was explored by comparing biomass formation (**Figure 2A**), media pH (**Figure 2B**) and recombinant protein yield (parts **C** and **D** of **Figure 2**), while culturing in BS medium and BS supplemented with different combinations of 50 mM phthalate (PL), 1% (w/v) yeast nitrogen base (YNB), and 1% (w/v) casamino acids (CA). Sypro-Ruby-stained gels loaded with equal volumes from day-5 culture supernatants were used to evaluate the relative effect on Hpf2-Pp accumulation (**Figure 2C**).

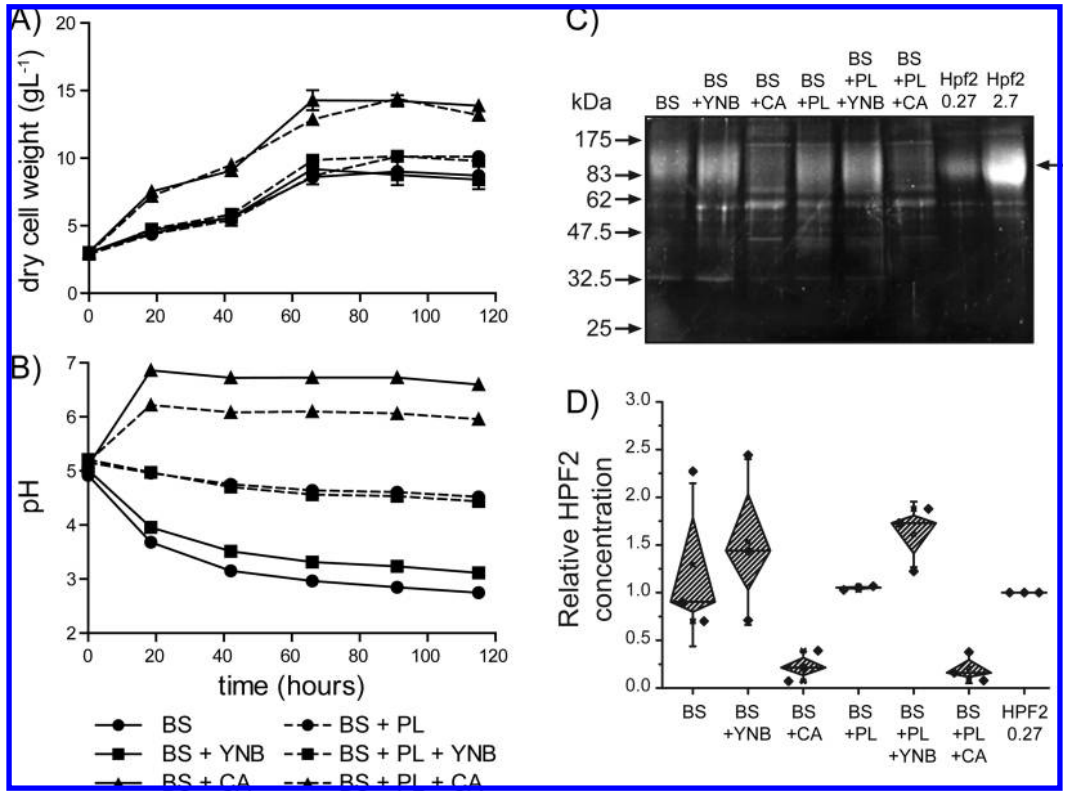

Figure 2. Two media supplements effect biomass accumulation, medium pH, and Hpf2 yield. Transformant Hpf2-Pp was grown under inducing conditions for 5 days in BS medium that had been supplemented with different combinations of YNB, CA, or PL. (**A**) DCW and (**B**) medium pH were recorded. Graphs show the mean of three replicate flasks, with error shown as 95% confidence intervals. (**C**) Representative gel loaded with equal volumes of media supernatants from day 5, separated by 10% (w/v) SDS−PAGE, and stained with Sypro Ruby. Hpf2 is indicated by an arrow on the right. The integral of Hpf2 intensity profiles was estimated from independent gels for each replicate and normalized to 0.27 μg of purified Hpf2. (**D**) Average normalized amount of Hpf2 produced by *P. pastoris* in each medium. The dots are values for each determination; the line across the diamond is the mean; the standard error is shown by the upper and lower ends of the diamond; and the standard deviation is shown by error bars.

As expected, unbuffered BS and BS + YNB media became increasingly acidic over the 5 days (**Figure 2B**). Phthalate (50 mM) provided sufficient buffering capacity to mitigate this pH change. However, the addition of 1% CA resulted in an increase in pH to 6.6. While the buffering capacity of phthalate was not sufficient to prevent the CA-induced pH increase, it did limit the increase to 6.0.

Biomass accumulation occurred during the entire methanol induction phase (**Figure 2A**). DCW increases of 5.9 $gL^{-1} \pm 0.4$ (BS), 6.2 $gL^{-1} \pm 0.5$ (BS + YNB), and 11.2 $gL^{-1} \pm 0.7$ (BS + CA) were observed in nonbuffered cultures. Increases of 7.2 $gL^{-1} \pm 0.2$ (BS), 7.3 $gL^{-1} \pm 0.2$ (BS + YNB), and 11.4 $gL^{-1} \pm 0.6$ (BS + CA) were observed in phthalate-buffered cultures. Biomass accumulation in CA-supplemented cultures was 5.0 ± 1.1 gL^{-1} greater than cultures in either the BS or BS + YNB media, irrespective of the addition of phthalate. Phthalate addition did have a small but statistically significant ($p < 0.01$) beneficial impact on biomass formation (1.4 ± 1.0 gL^{-1} increase) for cultures growing in BS and BS + YNB (**Figure 2A**). The observed increase in biomass was most likely due to the strong protection against pH decline provided by the buffer. The impact of phthalate was not evident for CA-supplemented cultures, for which media acidification did not occur.

Figure 2D shows normalized yields of Hpf2-Pp in culture supernatants. Maximal Hpf2-Pp yields were observed in BS or BS + YNB media. Minimal yield improvements and consistency were associated with phthalate and YNB supplementation, but these yield increases were not statistically significant. CA supplementation dramatically suppressed Hpf2-Pp production, despite it supporting much greater biomass accumulation. In previous studies, phthalate was added to provide buffering capacity against metabolism-induced medium acidification (*37*). In the present work, buffering of media with phthalate did prevent acidification and led to minor biomass yield improvements but was not associated with increased recombinant protein yield.

Purification of Hpf2 Produced in *P. pastoris*. Enrichment of Hpf2-Pp was required prior to use in haze protection assays because of the presence of non-Hpf2 protein in cell-free supernatants (lane 1 in **Figure 3**). Maximum accumulation of Hpf2-Pp in BMMY shake flask cultures was achieved by day 4, with little decline evident by day 5 (data not shown). Purification of Hpf2-Pp was achieved by the use of Ni^{2+}-affinity chromatography. Batch loading of resin following buffering and pH adjustment of cell-free supernatant was sufficient to bind the majority of recombinant protein (compare lanes 1 and 2 in **Figure 3**). Very little Hpf2-Pp was lost during column washing (lanes 3 and 4 in **Figure 3**). The inability to detect any other protein by silver staining indicates that largely pure protein was recovered by elution with 250 mM imidazole (lanes 5 and 6 in **Figure 3**). Ni^{2+}-Purified Hpf2-Pp was dialysed against water prior to lyophilization and use in haze protection assays. Three purifications yielded an average of 27.0 ± 0.8 mg of dry weight of purified recombinant protein per liter of culture.

Hpf2 Glycosylation by *P. pastoris*. The extent of Hpf2-Pp glycosylation was estimated by treating with endoglycosidase H (Endo H). This enzyme acts within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. Endo H treatment reduced Hpf2-Pp relative

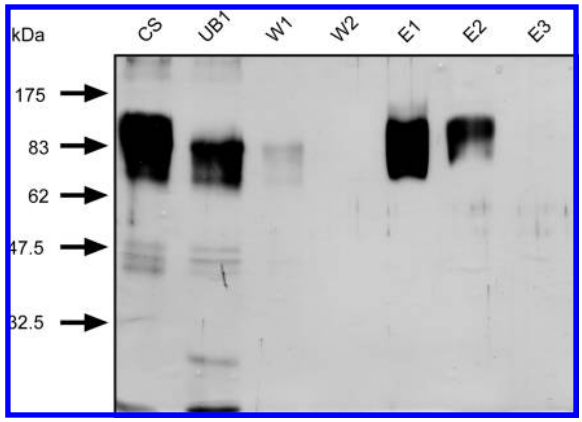

Figure 3. Purification of Hpf2-Pp. Fractions collected prior to and during nickel-affinity purification of Hpf2-Pp were separated by 10% (w/v) SDS−PAGE and protein visualized by silver staining. Culture supernatant (CS), unbound material (UB1), wash fractions (W1 and W2), and eluate fractions (E1, E2, and E3) are indicated above the image.

Table 1. Monosaccharide Linkage Analysis of Invertase, *S. cerevisiae*-Produced Hpf2, and *P. pastoris*-produced Hpf2

		mol %[a]		
monosaccharide	deduced linkage	inv.	Hpf2-Sc	Hpf2-Pp
Glc (p)	terminal-[b]	tr[c]	tr	1.0
	1,4-	−[d]	−	1.0
Man (p)	terminal-	41	42	54
	1,2-	19	25	25
	1,3-	5	5	tr
	1,4-	1	tr	1
	1,6-	9	2	2
	1,2,3-	tr	tr	tr
	1,2,4-	tr	tr	tr
	1,2,6-	22	19	5
	1,3,6-	2	4	9
	1,2,3,6-	tr	tr	tr
	1,2,4,6-	tr	tr	tr
GlcNAc	1,4-	tr	1	2

[a] mol % determined from the average of duplicate measurements. [b] Terminal Glc(p) deduced from 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, etc. [c] tr = trace, <1 mol %. [d] − = not detected.

molecular weight by approximately 40 kDa relative to untreated protein (lanes 4−6 in parts **B** and **C** of **Figure 1**). This compares to a reduction of ∼120 kDa following Endo H treatment of *S. cerevisiae*-derived Hpf2 (*12*). In the same study, it was also observed that Endo H treatment of a related protein, Hpf1, allowed purification by Ni^{2+}-immobilized-metal-affinity chromatography presumably because of increased accessibility of the histidine tag following mannose chain removal. Interestingly, Endo H treatment did not improve our ability to detect N-terminally tagged Hpf2-Pp by Western blot (**Figure 1C**), suggesting that any interference is most likely due to O-linked sugars.

The oligosaccharide structure of Hpf2-Pp was further investigated using monosaccharide linkage analysis. A comparison between Hpf2, Hpf2-Pp, and invertase is shown in **Table 1**. The linkage analysis shows an increase of approximately 10 mol % in the proportion of terminal mannose and an overall 10 mol % reduction in oligosaccharide branching in Hpf2-Pp compared to *S. cerevisiae*-produced protein. Hpf2-Pp also exhibited a shift in the proportion of branch types, with the protein differing predominantly in levels of 1,2,6-linked mannose, which was greatly reduced, compared to Hpf2. As expected, 1,3-linked mannose was undetectable in the *P. pastoris*-produced form (*38*). Conversely, the proportion of

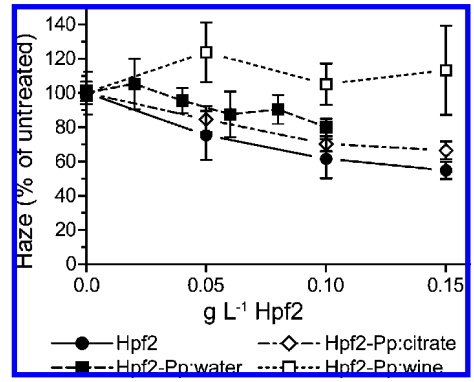

Figure 4. Haze protective activity of Hpf2-Pp. Haze-reducing activity of *P. pastoris*-produced Hpf2 (Hpf2-Pp) was compared to *S. cerevisiae*-produced Hpf2 (Hpf2). Freeze-dried protein was resuspended in either water, wine, or citrate and used to treat wine at the indicated concentrations. Treated samples were subjected to a heat test. The degree of haze formation is expressed as a percentage of haze formed in untreated wine. Data points represent the average of four replicates, with error bars indicating 95% confidence intervals.

1,3,6-linked mannose was greater in Hpf2-Pp compared to either Hpf2 or invertase. Overall, these results indicate that mannose chains in Hpf2-Pp were both shorter and less branched than those in Hpf2.

Haze Protective Activity of *P. pastoris*-Produced Hpf2. The ability of Hpf2-Pp to reduce protein haze in white wine was compared to the *S. cerevisiae*-produced form. Previous investigations of Hpf2 have used aqueous solutions of Hpf to evaluate haze reductive activity (*12*). The *P. pastoris*-produced protein had very limited solubility in water (limit < 1 gL^{-1}). Therefore, solutions of Hpf2-Pp prepared in water, wine, and citrate buffer (20 mM at pH 4.0) were tested for their ability to reduce haze in an attempt to maximize the amount of Hpf2-Pp in the heat test. **Figure 4** shows the effect of Hpf2-Pp addition on the heat stability of white wine in comparison to the addition of an aqueous solution of Hpf2. Hpf2-Pp solubilized directly in wine had no stabilizing effect. Both aqueous and citrate-buffered solutions showed similar trends with the maximum protective effect observed with the addition of 0.15 gL^{-1} Hpf2-Pp (final concentration) citrate solution, resulting in a 30% reduction in haze formation. This compared to a 45% reduction in haze formation when aqueous solutions of Hpf2 were used at the same (w/v) dosage rate. Taking into account different average molecular weights of the two glycoproteins, Hpf2-Pp has lower haze reducing capacity, resulting in a 19% haze reduction of μmol^{-1} L^{-1}, compared to a 54% haze reduction μmol^{-1} L^{-1} for Hpf2.

Haze Protective Activity of Hpf2 Produced in Mutants of *S. cerevisiae*. The role of glycan structure in haze protection was further investigated by producing Hpf2 in different *S. cerevisiae* backgrounds with deletions of *MNN1*, *MNN2*, *MNN4*, and *MNN5*. These genes encode mannosyltransferases involved in α-1→6 outer glycan chain decoration (*39, 40*). Complete elimination of haze protective activity was observed for Hpf2 produced in a *mnn2*-Δ background (**Figure 5**). Strains carrying *mnn2*-Δ produce glycoproteins with an unsubstituted, α-1→6-linked backbone of the outer chain attached to a wild-type core oligosaccharide (*39*). A small but significant contribution ($p < 0.05$) to haze protection was related to phosphomannose addition demonstrated by the reduction in haze protection observed when Hpf2 was produced in a *mnn4*-Δ background. Interestingly, neither α-1→3 mannosylation nor further α-1→2 branch elongation contributed to haze protective activity. In fact, the

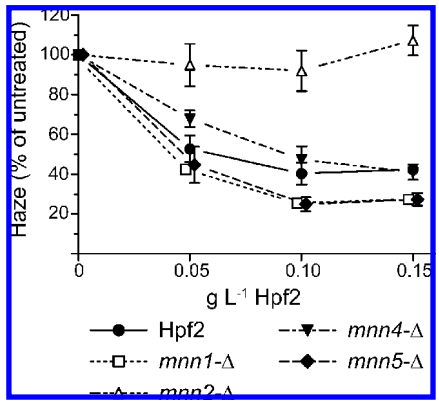

Figure 5. Haze protective activity of Hpf2 is altered in proteins with different glycosylation profiles. Hpf2 was produced in wild-type *S. cerevisiae* (Hpf2) or *S. cerevisiae* carrying deletions in either *MNN1* (*mnn1*-Δ), *MNN2* (*mnn3*-Δ), *MNN4* (*mnn4*-Δ), or *MNN5* (*mnn5*-Δ). Freeze-dried, purified Hpf2 was resuspended in water and added to wine at the indicated concentrations. Treated samples were subjected to a heat test. The degree of haze formation is expressed as a percentage of haze formed in untreated wine. Data points represent the average of four replicates, with error bars indicating 95% confidence intervals.

increased protection against haze provided by Hpf2 produced in *mnn1*-Δ and *mnn5*-Δ backgrounds suggests that these modifications are limiting (**Figure 5**).

DISCUSSION

It has previously been noted that levels of haze protective mannoproteins expressed naturally under winemaking conditions are too low to be of commercial significance (*16*). Alternative mannoprotein production methods need to be investigated if their use in a commercial setting are to be viable (*12*), as is a greater understanding of their mode of operation and their sensory impact. This study examines the possibility of using a commercial expression host for mannoprotein production and how the altered mode of production impacts the effectiveness of the molecule as a haze protective factor.

Recombinant Hpf2 was successfully produced and secreted in *P. pastoris* by fusing the main open-reading frame to the *S. cerevisiae* α-factor signal peptide (*41*). Two 6× His-tag positions (N- and C-terminal) were assessed. Both arrangements supported strong Hpf2 production and secretion according to silver staining. A GPI anchor sequence predicted in the N-terminally tagged protein did not appear to affect protein release into the medium. However, the N-terminally tagged version, Hpf2NHis, could not be efficiently detected by Western blot, and it was for this reason that subsequent work was performed using C-terminally tagged protein. Given that endoglycosidase H treatment of Hpf2NHis did not improve its detection by anti-tetra-His antibody, we speculate that Hpf2 may be substantially O-glycosylated in the vicinity of its N terminus.

On the basis of previous reports, a number of media additives were evaluated for their impact on Hpf2-Pp production. Hahn-Hägerdahl et al. (*37*) described the beneficial impact of 50 mM phthalate in relation to its buffering capacity and effect on biomass formation during aerobic growth in both complex and defined media. In the work described here, phthalate was used in defined medium only but was tested in combination with other additives. The addition of phthalate was associated with the increased stability of culture media pH and small increases in biomass accumulation compared to nonbuffered cultures but did not lead to measurable increases in recombinant protein yield. Much greater increases in biomass accumulation were observed with 1% (w/v) casamino acid supplementation. However, casamino acid use was also associated with a large increase in medium pH, against which phthalate did not provide sufficient buffering capacity.

Contrary to its effect on biomass formation, a strong suppressive effect on Hpf2-Pp yield was observed with casamino acid supplementation. A similar suppression of recombinant protein production by casamino acid use was noted by Chen et al. (*35*). These findings are in contrast with other studies, in which use of this supplement resulted in increased yields of secreted recombinant proteins, in particular, EGF (*41*), MSP3 (*42*), and ABP-CM4 (*43*). Clare et al. (*41*) and Zhang et al. (*43*) attributed protein yield increases to suppression of proteolysis; however, in these cases, the medium was simultaneously buffered to a pH of 6.0. Jahic et al. (*44*) were unable to detect any reduction of protease activity related to the use of casamino acids. Wang et al. (*42*) also found little correlation between MSP3 yield and protease production. Casamino acid supplementation resulted in increased MSP3 yields, whereas ammonium or amino acid cocktail supplementation had the opposite effect. In all of the studies above, the recombinant proteins produced were optimally expressed at pH 6 or above. It is thus possible that casamino acid supplementation is acting as a buffer, effectively preventing respiration-associated pH decline, and thus may be beneficial only for proteins for which a high pH condition is ideal.

A comparison of *P. pastoris*- and *S. cerevisiae*-produced Hpf2, by analysis of their glycosylation profile and their capacity to inhibit haze formation, has demonstrated differences in both attributes. Treatment with Endo H showed mannose-linked structures contributed between 35 and 45 kDa to the molecular weight of Hpf2-Pp compared to 120 kDa for Hpf2 (*12*). A similar contribution to protein size by mannose was reported for invertase produced in *P. pastoris* (*45*). The major structural differences between glycan chains of *P. pastoris*- and *S. cerevisiae*-produced Hpf2 were 1,2,6 linkages, which were reduced 5-fold as a proportion of total mannose in Hpf2-Pp compared to Hpf2 or invertase. An increase in the proportion of terminal mannose was also observed, which is consistent with a reduction in the chain length of *P. pastoris* oligosaccharides compared to *S. cerevisiae* and is well-documented in previous studies (*46*, *47*). A complete absence of 1,3 linkages was also observed, reflecting the absence of 1,3 mannosyltransferase in *P. pastoris* (*38*).

The altered glycosylation profile impacted on the physical properties of Hpf2-Pp and its capacity to provide protection against protein haze formation in white wine compared to Hpf2. Hpf2-Pp solubility was reduced, with a solubility limit of less than 1 gL^{-1}. This affected our ability to test the haze-reducing capacity of Hpf2-Pp at higher concentrations. Nevertheless, the per molecule protein haze inhibition capacity of Hpf2-Pp was estimated to be approximately 2.8-fold less than that of Hpf2. The reduced haze protective capacity of Hpf2-Pp demonstrates the importance of glycan outer chain length in haze prevention. However, Hpf2 produced in *S. cerevisiae mnn2*-Δ demonstrates that long outer chains are not in themselves sufficient to confer haze protective activity on Hpf2. The addition of mannose by α-1→2 linkage to the α-1→6 outer chain is both necessary and sufficient for full haze protective activity of Hpf2. Thus, the shorter outer oligosaccharide chain of *P. pastoris* glycoproteins and, hence, fewer overall α-1→2 branches may explain the reduced haze protective activity of Hpf2-Pp.

Other glycoproteins with haze protective activity in wine, a wine arabinogalactan-protein (AGP; *48*), gum arabic, and an apple arabinogalactan-protein (*49*), also have a relatively high proportion of carbohydrate to protein. The glycosylation pattern of the wine AGP was modified using enzymatic and chemical treatments (*48*). These studies led to similar conclusions as those above: branching of the glycan chains was important to haze protective activity.

Glycosylation may have a direct role in haze protective activity by providing an "active" site for interaction with haze-forming proteins or other wine components; it may have an indirect role by maintaining stability of the Hpf2 peptide backbone; or it may have a shared role with both the glycan and protein backbone acting together as an "active" site. While it is known that wild-type Hpf2 survives both heat treatment and the action of proteases in wine, the stability of Hpf2 with altered glycosylation profiles is not known. Evaluation of the impact of glycan structure on Hpf2 stability will be important if this class of proteins is to be employed commercially in the wine industry and may provide insights into the relationship between Hpf2 stability and function. *P. pastoris* is not a suitable production host for Hpf2 because of the importance of glycan structure for Hpf2 activity and the reduced activity and limited solubility of Hpf2 when produced in this organism.

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